CELL VIABILITY AND MORPHOLOGICAL EFFECT OF *Heterotrigona itama sp.* (STINGLESS BEE) HONEY ON MALIGNANT BRAIN TUMOUR CELL LINE.

By

PRIATHARSINE SEERANGAN

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ANALISIS KEMANDIRIAN DAN KESAN MORFOLOGI MADU *Heterotrigona itama sp.* (LEBAH KELULUT) TERHADAP SEL SELANJAR TUMOR OTAK MALIGNAN.

Oleh

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Tesis diserahkan untuk memenuhi sebahagian keperluan bagi

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Introduction: Glioblastoma Multiforme (GBM), a type of primary brain tumor is found to account for almost 80% of these tumors and, therefore, signifying the frequent occurrence. In Malaysia, a constantly increasing incidence along with limitations on currently available therapy options had urged the need for newer approaches to be implemented. For centuries, honey, a sweet natural product has been described in traditional medicine for its various medicinal uses. However, until recently honeys have been both researched scientifically and exploited medicinally on its various kinds of photochemical constituents.

Objectives: The study is aimed to evaluate the anticancer activity of honey sample produced by a stingless bee species (*Heterotrigona itama sp)* for morphological analysis and cytotoxic activity against malignant brain tumor cells to provide evidence on its anticancer effect.

Methods: Human primary glioblastoma *cell line;* U87 and dBTRG, are grown exponentially and treated with honey concentrations of 2-10% for three sequential days (24h, 48h, 72h). Honey sample from the stingless bee *Heterotrigona Itama sp.* were collected under sterile condition. The cytotoxicity of honey is quantified using colorimetric MTS assay with treatment of 2-10% of stingless bee honey. The plates were incubated and absorbance value was measured using ELISA reader at 490nm. For morphological analysis, the cell lines were seeded into six-well plates, and treated with 2% to 10% concentration of

the honey for 24, 48 and 72 hours. At the indicated time points, morphological changes were examined and recorded under light microscope**.** Apoptosis was determined at similar intervals and concentrations after staining the cells with a nucleic acid stain known as Acridine Orane/Propidum Iodide.

Results: Overall, crude honey sample have higher cytotoxic activities on U87 than dBTRG cell lines in a dose dependent manner, with U87 cell line showing higher sensitivity towards the honey extract. For morphological analysis, honey treated cells exhibited morphological alteration such as nuclear shrinkage, chromatin condensation and fragmented nucleus indicating apoptotic cellular changes.

Conclusion: In conclusion, *Heterotrigona Itama sp.* contains an *in vitro* cytotoxic activity against human glioma cell lines, therefore suggesting the anticancer effect of the stingless bee honey. Further study is however required which includes isolation and characterization of the anticancer agents.

Supervisor: Dr Farizan Ahmad

Co-Supervisor: Dr Zulkifli Mustafa

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Pia…

CHAPTER 1

INTRODUCTION

1.1 Study background

Two percent of human malignancies are by primary tumor of central nervous system (CNS). Malignant gliomas/glioblastoma (GBM) known as one of the most aggressive neoplasm, had been the most commonly occurring CNS tumors. The annual incidence of malignant gliomas is approximately 5 cases per 100,000 people (WHO, 2008). It's incidence in Malaysia had shown to constantly increase through the years with GBM contributing the most (Yusoff *et al.*, 2004). Generally, malignant gliomas are not curable and although relatively uncommon, malignant gliomas are associated with disproportionately high morbidity and mortality. In the past two decades tumor management have improved in a large scale in terms of novel approaches such as surgery, radiation and chemotherapy. These advances have led for a better quality of patients' life in variable degrees and survival improvement (Jones and Holland, 2010).

Glioblastoma (GBM) is one of the most common and lethal primary brain tumor which demonstrates a high proliferation rate, aggressive growth pattern and largely resistant to chemotherapy (Agnihotri *et al.*, 2013; Gangemi *et al.*, 2009). With over 40 years of research in this area, there is only a little improvement seen in mortality rates among patients diagnosed with GBM. This has been the case due to the intra- and inter-tumoral heterogeneity and the tendency of the cancerous cells to infiltrate into the normal brain parenchyma. Besides these, blood-brain barrier prevents chemotherapeutic agents from adequately penetrating into the tumor mass and leads to suboptimal therapeutic response had further complicated the scenario (Stummer *et al.*, 2011). Therefore, it has always been a challenge for every researcher to come up with a solution in providing effective therapies for the treatment of GBM, dilemma evidenced in median survival of 15 months and remained practically unchanged over the past several decades (Bleeker *et al.*, 2012).

Currently, researchers are seeking for new substances that may reduce the viability of cancer cells, slow tumor growth, and extend life expectancy. Therefore, increased interests have been subjected against natural products for their importance in sustaining good life of human by preventing or treating cancer. Among the apicultural products that possess functionality characteristics (antibacterial, anti-inflammatory, antiviral), honey has been widely presented in various culture cell lines (Barbarić *et al.*, 2011; Borges *et al.*, 2011; da Silva Frozza *et al.*, 2013). Honey can be obtained from honey bees as well as stingless

bees. Stingless bees are known as highly eusocial insects with over 500 species in 32 genera with probability of 100 more new ones yet to be identified and characterized (Michener, 2000; Michener, 2013).

Stingless bees produce precious products such as honey, which is stored in propolis-rich cerumen pots. Compounds such as polyphenols (phenolic acids, flavonoids and their derivatives), terpenes, steroids, and amino acids are particularly interesting in which they are considered as an important part of traditional medicine (Küçük *et al.*, 2007). Previous study by Jaganathan and colleague had reported a strong cytotoxicity of honey against U87 cell line which suggested at possibility of connection between the polyphenols content in the honey (Jaganathan and Mandal, 2009b). The paper also proposed that polyphenols in honey such as caffeic acid, caffeic acid phenyl ester, chrysin, galangin, quercetin, kaempferol, acacetin, pinocembrin, pinobanksin and apigenin may be responsible in the anti-proliferative activity observed. Despite that, other biochemical compounds found in honey such as the chrysin can be used to prevent cancer similar to the action of a breast cancer drug (anastrozole) (Galijatovic *et al.*, 2001). However in another study, a dose dependent decrease in cell viability of glioma cells was suggested to be caused by Lead (Pb) contained in the honey, where Pb was suggested to activate p38MAPK and JNK1/2 to start a series of effect in the C6 glioma cells (Posser *et al.*, 2007).

Effect of caffeic acid and its ester present have also been proven to play a vital role in mechanism of antiproliferative and apoptotic effects on malignant cells in various studies done. Rao and colleagues (1992), performed a detailed study by synthesizing caffeic acid esters and examined them against 3, 2 -dimethyl-4-aminobiphenyl (DMAB, a colon and mammary carcinogen). Another study reported on strong repressive effect of CAPE, in which inhibitory effect of CAPE was established on TPA-induced tumor promotion by topical application of CAPE in CD-I mice (Huang *et al.*, 1996). A study done in Iran, reported that honey is capable of inducing apoptosis in bladder cancer cell lines (T24, RT4, 253J and MBT-2). Ability of honey to inhibit proliferation in these cell lines were also showed by using 1-25% of honey concentration, which further clarified that honey possesses potential antitumor known as antimetastatic effects (Samarghandian *et al.*, 2011). Another similar study conducted in Brazil using *Tetragonisca fiebrigi* stingless bee, has revealed that the bee product exhibits antioxidant as well as anti-inflammatory activities. Cytotoxic activity was also determined in a concentration-dependent against K562 cells (Campos *et al.*, 2014). Therefore, these studies suggested for possible importance of the stingless bee species honey product in as cancer treatment.

To our knowledge, anticancer activity of stingless bee (*Heterotrigona itama sp.)* has not yet been analyzed. The present study has been chosen to investigate for the first time, the cytotoxic effect of *Heterotrigona itama sp.* honey product on human brain cell line. This study is expected to develop the present information

on the chemical characterization, anticancer activity of the stingless bee honey and to assist in a more focused design of further research, such as aiming at more specified applications of this product as a natural adjuvant treatment against GBM. Stingless bees' honeys comprising sugar and water for 95% of the honey dry weight along with numerous compounds such as organic acids, proteins, amino acids, minerals, polyphenols, vitamins (ascorbic acid) and aroma compounds. Pharmacologically active components, especially flavonoids and phenolic constituents are thought to contribute towards medicinal benefits of these stingless bee honeys (Bogdanov *et al.*, 1996).

This study is aimed to investigate the anti-proliferative as well as pro-apoptotic effects of stingless bee honey on two malignant glioma cell lines; (glioblastoma multiforme – U87MG and glioma cell with high degree of resistance: dBTRG). Additionally, morphological analyses which include light and fluorescence microscopy methods were performed.

1.2 Rationale of study

Current treatments for GBMs are with several limitations such as difficulties to achieve complete surgical resection, failure in precise drug delivery and also side effects which accompany the radiation and chemotherapy (Bonavia *et al.*, 2011; Kesari, 2011). Thus newer therapeutic strategies are in urgent need. Failing in removing the tumor completely through surgery will cause recurrent cases which therefore require adjuvant therapies such as radiation and chemotherapy. However, studies shows that though the tumor itself has most deleterious effect on cognitive function, radiotherapy may result in additional long term effects when high fraction dose is applied as the treatments could not discriminate between normal and cancer cells (Auffinger *et al.*, 2012). In some cases, cells are becoming resistant to radiation therapy; such that upon clinically approved dosage use, tumors could not being eradicated successfully (Baumann *et al.*, 2009).

Moreover, long term use of chemotherapy drugs such as Temozolomide (TMZ). Several studies have reported the roles played by these resistance mechanisms in causing cancer resistance against the chemotherapeutic drugs assigned (Ma *et al.*, 2010). For example, TMZ had been associated with thrombosis and bleeding problems (Patel *et al.*, 2013). Other side effects associated with these drugs are also devastating such as seizure, numbness, and signs of infections, easy bruising and bleeding, loss of appetite, nausea, vomiting and fatigue. Due to this

condition, there is an urgent need to find newer anticancer agents to be developed into a novel anticancer drug which is able to evade the resistance mechanism and unfavourable side effects.

Among the many herbs and natural products being targeted for these purposes, honey is found to be an interesting source of few bioactivities. For examples, several studies have shown anticancer properties of stingless bee honey and its phenolic components by different mechanisms such as cell cycle arrest as in inhibition of cancer cell proliferation in colon cancer (Jaganathan and Mandal, 2009a), glioma (Lee *et al.*, 2003) and melanoma cancer cell line in G0/G1 phase (Pichichero *et al.*, 2010), induction of apoptosis (Jaganathan and Mandal, 2009a), induction of mitochondrial stress(Jaganathan and Mandal, 2010), effects on tumor necrosis factor (Tonks *et al.*, 2003), anti-inflammatory and immunomodulatory activities (Attia *et al.*, 2008) and anti-mutagenic activity (Saxena *et al.*, 2012). Several reports have been made on anticancer activity of stingless bee honey from various parts of the globe. Though most of the studies were done using the honeybee product (*Apis sp*), suggesting their various bioactivities such as anti-proliferative and pro-apoptotic activity. Little attention had been projected towards stingless bee product though stingless bee honeys are still being used in traditional medicine in several places such as in Central and South America, and Africa (Cortopassi-Laurino *et al.*, 2006). Several studies had suggested the likelihood of therapeutic properties that may be held by stingless bee honey which are similar to presently used curative honeys such as honeybee (Adams *et al.*, 2008; Borges *et al.*, 2011; Cooper *et al.*, 2000). Other examples

include antimicrobial and antiproliferative activities of *Tetragonula laeviceps sp.* reported in Thailand (Umthong *et al.*, 2011; Umthong *et al.*, 2009) and *Melipona scutellaris* sp. from Brazil with similar anticancer properties (da Cunha *et al.*, 2013). Therefore, these interesting reports prompted us to study the anticancer potential of *Heterotrigona itama sp.* stingless bee honey, which is found in Kelantan, Malaysia.

1.3 Objectives of the study

1.3.1 General Objective:

To study the anticancer effects of *Heterotrigona itama sp.* honey on human GBM cell lines.

1.3.2 Specific Objectives:

1. To evaluate the effect of *Heterotrigona itama sp.* honey on cell viability of human GBM cell lines.
2. To visualize the effect of *Heterotrigona itama sp.* honey on morphological changes of human GBM cell lines.

CHAPTER 2

LITERATURE REVIEW

2.1 Malignant glioma (Glioblastoma, GBM)

Cancers are some of the most fatal diseases known to human kinds. Tumors can either be malignant or benign, whereby benign tumors are usually harmless to body unless it is increasing in mass and start pressing on nearby internal organs and tissues (Louis *et al.*, 2007). An example would be brain tumors, which have generally stricken deep into psyche of those receiving and those delivering the diagnosis. It includes both neoplasms emerging directly from brain (primary brain tumor) and those involving the brain as metastatic site (secondary brain tumor) which are capable of indirectly damaging healthy cells by crowding other parts of the brain, therefore causing inflammation, brain swelling and pressure within the skull (Omuro and DeAngelis, 2013). Sixty percent of the tumors originate from glial cells (glioma tumor). GBM is a type of malignant brain tumor arising from the glial cells of the brain which are responsible in surrounding and supporting the brain neurons. These tumor cells grow uncontrollably by disobeying normal cell division rules as well as rapidly

reproducing, in a restricted nutrient and spaces to grow resulting in invasion and metastasis (Agnihotri *et al.*, 2013).

2.1.1 Glioma classification

Presence of both neoplastic and stromal tissues in GBM had contributed towards both histologic heterogeneity and variability in outcomes. This therefore requires a better classification of these tumors using molecular studies such as gene-expression profiling and aids in classification of these tumors to various prognostic groups (Wen and Kesari, 2008). In general, glial tumors are distinguished by means of their cell type, by location and by grade. Classification according to cell type are through close resemblance of which cell they represent the most leading to several types such as ependymoma (ependymal cells), astrocytomas (astrocytes), oligodendrogliomas (oligodendrocytes) (Huttner, 2012). GBM are also classified depending on their location whether above or below brain membrane called tentorium. The membrane acts as separation of the cerebrum and cerebellum, leading to classification such as supratentorial (above tentorium) and infratentorial (below tentorium). World Health Organization (WHO) had classified higher grade glioma as grades III and IV based on tumor histological and immunohistochemical features (Louis *et al.*, 2007). Much specifically, anaplastic astrocytomas, anaplastic oligodendrogliomas, and anaplastic oligodendroastrocytomas are classified as grade III while GBM and other less common variants are classified as grade IV. Glioma classification is

illustrated below in Table 1.1. GBMs, being the most aggressive and deadly of these tumors had thus become the most common cancer of the central nervous system (CNS) accounting for 53% of gliomas overall (Huse and Holland, 2010). Lower grade gliomas such as the grade 1 and 2 (benign) are with a better prognosis compared to higher grade gliomas. However, lower grade gliomas often have higher likelihood of emerging to grade 4 type GBM (Jones and Holland, 2012).

Table 1.1: World Health Organization (WHO) classification of common glial tumors in humans. Adapted from (Phillips *et al.*, 2006).

	WHO grade	Tumor histology	Subtype by histology
Low grade	I	Benign, well circumscribed.	Pilocytic astrocytoma
	II	Diffuse infiltration, low proliferation	Diffuse astrocytoma Oligodendroglioma Oligodendrocytoma
High grade	III	Highly infiltrative, high proliferation.	Anaplastic-astrocytoma Anaplastic-oligoastrocytoma Anaplastic-oligodendroglioma
	IV	Highly infiltrate, high vascular and Cellular proliferation.	Glioblastoma-Multiforme

2.1.2 Incidence, Symptoms

Annual incidence of glioma is approximately 5 cases per 100,000 people (WHO, 2008), while, it's incidence in Malaysia had shown to constantly increase through the years with GBMs being the most frequently repeated cases (Yusoff *et al.*, 2004). The average age of diagnosis is 65 and found to be common in men as compared to women. Low survival rate of GBM patients, ranging between 12 to 14 months which had made GBM as an urgent subject of cancer research. GBM accounts for approximately 60 to 70% of malignant gliomas, 10 to 15% of anaplastic astrocytomas and 10% of anaplastic oligodendrogliomas and anaplastic oligoastrocytomas. Less common tumors such as anaplastic ependymomas and anaplastic gangliogliomas account for the rest (Wen and Kesari, 2008). The incidence of these tumors has increased slightly over the past two decades especially in the elderly, primarily due to improvements in diagnostic imaging (CT and MRI), which have enabled us to detect the tumors in advance. Moreover, availability of more neurosurgeons, precise characterization of the tumors and also changes in therapy approaches have attributed to elevated incidence of detectable cases (Schwartzbaum *et al.*, 2006). Symptoms for GBMs are similar to other malignant brain cancers, which include headache, seizure, memory loss, and physical weakness such as loss of muscle control, visual symptoms, language problems and cognitive decline (Behin *et al.*, 2003). The exact cause of GBM is still inconclusive. However, 5% of the tumors have known hereditary factors. Genetic alterations in glioma have been correlated with

pathways of cancer hallmarks such as cell proliferation, cell survival, invasion and angiogenesis (Furnari *et al.*, 2007; Hanahan and Weinberg, 2011).

2.1.3 Molecular pathogenesis

GBM are generally classified into two subtypes (primary GBM and secondary GBM) in reference to their biologic and genetic differences. Frequently occurring in elderly patients, primary GBMs are featured by EGFR amplification and mutations, loss of heterozygosity of chromosome 10q, deletion of the phosphatase and tensin homologue on chromosome 10 (PTEN), as well as p16 deletion (Wen and Kesari, 2008). Whilst, secondary GBM often occurring in younger patients, are characterized by mutations in the p53 tumor suppressor gene (TSG), overexpression of the platelet derived growth factor receptor (PDGFR), abnormalities in the p16 and retinoblastoma (Rb) pathways, and loss of heterozygosity of chromosome 10q. Both primary and secondary subtypes of GBM differ from each other in terms of transcriptional patterns and aberrations in the DNA copy (Wen and Kesari, 2008).

2.1.4 Current treatments

Standard treatments applied for GBMs are maximum resection of the tumor by surgery, chemotherapy using drug known as temozolomide and radiotherapy. Though malignant gliomas cannot be completely eliminated surgically because

of their infiltrative nature, patients should undergo maximal surgical resection as possible to ensure complete elimination of the tumor (Steiger *et al.*, 2000). Conversely, due to heterogeneity of the tumor, goal of these treatments are hard to achieve and in almost 80% of the cases there will be recurring tumor (Olar and Aldape, 2014). Patients' survival rate in GBMs is also very poor and had not shown any improvement for the past 20 years. Thus, urge to develop therapies for brain cancers in neuro-oncology field was put up front in hope for improvement of the patients' survival rate. To test the experimental therapies, most of the studies require the use of animal as well as in vitro models of brain tumor.

2.1.5 Alternative treatment options

Chemotherapy consisting drugs such as procarbazine, lomustine and vincristine is known as the most common combination regime treating higher grades gliomas. Additionally, carmustine-polymer wafers were also approved by FDA in 2002, where the wafer would be placed at the tumor location for initial treatment (Bota *et al.*, 2007). Survival rate is higher in those underwent radiation therapy than patients who did not (Stupp *et al.*, 2002). Adding up to the evidence, final result of randomized phase 3 trial on prolonged survival rate on those received adjuvant temozolomide with radiotherapy has also been reported (Stupp *et al.*, 2005). Patients with methylated MGMT promoter adjuvant with chemotherapy revealed longer survival rate (Hegi *et al.*, 2005). However, lesser

aggressive treatments are opted for patients of 70 years and above (Ahmed *et al.*, 2014).

2.2 Honey

Honey is one of the oldest best loved sweetening agents produced by honeybees and stingless bees. Use of natural honey as food and medicine products has long been present from time immemorial by ancient Egyptians, Assyrians, Greeks and Romans in which they used honey for wounds and diseases of intestine (Bent, 2013; Dhinsa *et al.*, 2013; SHARMA *et al.*, 2012). Though honey has been long appreciated for its medicinal properties in the earlier days, its use in modern medicine is very much limited due to restricted amount of studies and scientific supports (Mirzaie *et al.*, 2012). However, in the past decade, benefits of honey had become more dedicated towards its curative properties, few examples of most remarkable discoveries made are antibacterial (Alvarez-Suarez *et al.*, 2010; Jeffrey and Echazarreta, 1996), antioxidant (Hegazi and Abd El-Hady, 2009), anti-inflammatory (Medhi *et al.*, 2008; Tonks *et al.*, 2003) and anticancer properties (Al-Mamary *et al.*, 2002; Ghashm *et al.*, 2010; Swellam *et al.*, 2003; Wen *et al.*, 2012). Moreover in recent study, also revealed anti-cancer property of Malaysian jungle honey on various cell lines such as human breast cancer, cervical, oral and osteosarcoma cell lines (Fauzi *et al.*, 2011). Anti-proliferative property was reported in several papers (Fernandez-Cabezudo *et al.*, 2013; Samarghandian and Samini, 2014). Honey is therefore classified into both

nutraceutical and therapeutic food categories, meaning that honey is capable of serving sufficient quantities of nutrients to satisfy particular organic needs, thus reducing risks of developing several illnesss.

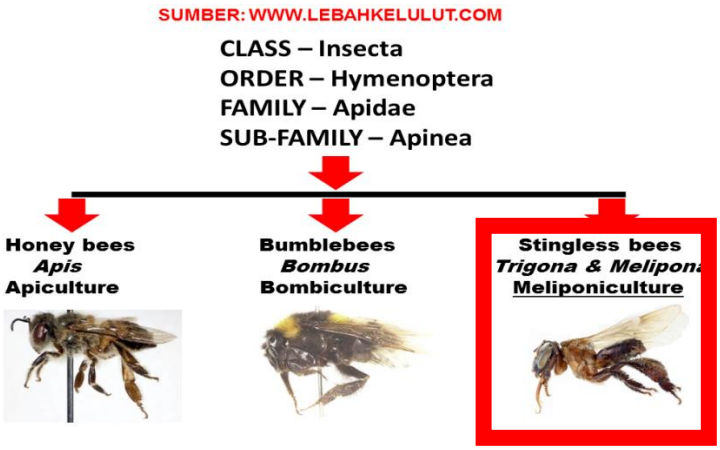
2.2.1 *Heterotrigona itama sp.* (stingless bee)

With majority of studies are focusing on honey produced by the honeybee, little attention has been given towards honey obtained from stingless bees. Currently, stingless bee honeys are still being used in traditional medicine in several places such as in Central and South America, and Africa (Cortopassi-Laurino *et al.*, 2006). Increasing evident had suggested the possibility of therapeutic properties that may be held by stingless bee honey which are similar to currently used medicinal honeys such as honeybee (Adams *et al.*, 2008; Cooper *et al.*, 2000; George and Cutting, 2007). Therefore, the main goal of using *Heterotrigona itama sp* (honey product) in our current study is to examine potential anti-cancer property of the stingless bees, which is available in Malaysia and commonly manufactured by local bee keepers. To our knowledge, this is the first study to evaluate the bioactivities of *Heterogena itama sp.*

Belonging to five different genera (Melipona, Trigona, Meliponula, Dectylurina and Lestrimelitta), the stingless bees are a group of eusocial insects which plays a vital role in pollination process (Heard, 1999). Trigona is the largest genus of stingless bees, formerly including many more subgenera than the present

assemblage; many of these former subgenera have been elevated to generic status (Gupta, 2014). There are approximately 150 species presently included in the genus, in 11 subgenera. They differ from those groups now excluded in only minor structural details, primarily of the hind leg. Meliponines, are a large group of bees, comprising the tribe Meliponini (sometimes called stingless honey bees) in the family Apidae, and closely related to the common honey bees, carpenter bees, orchid bees and bumblebees. Stingless bees usually nest in hollow trunks, tree branches, underground cavities, or rock cavities, old rubbish bins, water meters, and storage drums. Many beekeepers keep the bees in their original log hive or transfer them to a wooden box, as this makes it easier to control the hive (Vit *et al.*, 2013).

(a)



(b)

Figure 2.1: (A and B) Shows stingless bee *Trigona* image (top right) and method of stingless bee honey acquisition from its pots filled with honey (bottom) Adapted from www.lebahkelulut.com.

2.2.2 Medicinal properties of stingless bee honey

Lack of stingless bee honey quality standards resulted in no publishing on quality control declaration for consumers, however had encouraged more studies using stingless bee products to be done (Chuttong *et al.*, 2016).

Stingless bees' honeys are known to have pharmacologically active components which are thought to be contributing to the medicinal benefits of these honeys. Sugars are the main constituents, comprising about 95% of the honey dry weight. Besides sugars and water, the honey contains numerous compounds such as organic acids, proteins, amino acids, minerals, polyphenols, vitamins (ascorbic acid) and aroma compounds (Bogdanov *et al.*, 2008; Chuttong *et al.*, 2016). Particularly interesting are compounds such as polyphenols (phenolic acids, flavonoids and their derivatives), terpenes, steroids, and amino acids, which are considered as an important part of traditional medicine. Most imperative compounds being studied are phenolic acids and flavonoids, which are believed to contribute to the high antioxidant activity in the honey (Küçük *et al.*, 2007). A review paper published by Othman (2012) also suggests that therapeutic properties of honey are capable of counteract the multi-factorial causes of carcinogenesis (excess free radical production, chronic infection, chronic inflammation, decreased immune level, chronic ulcers and hereditary). The inverse relationship between honey and cancer is illustrated in Figure 2.2.

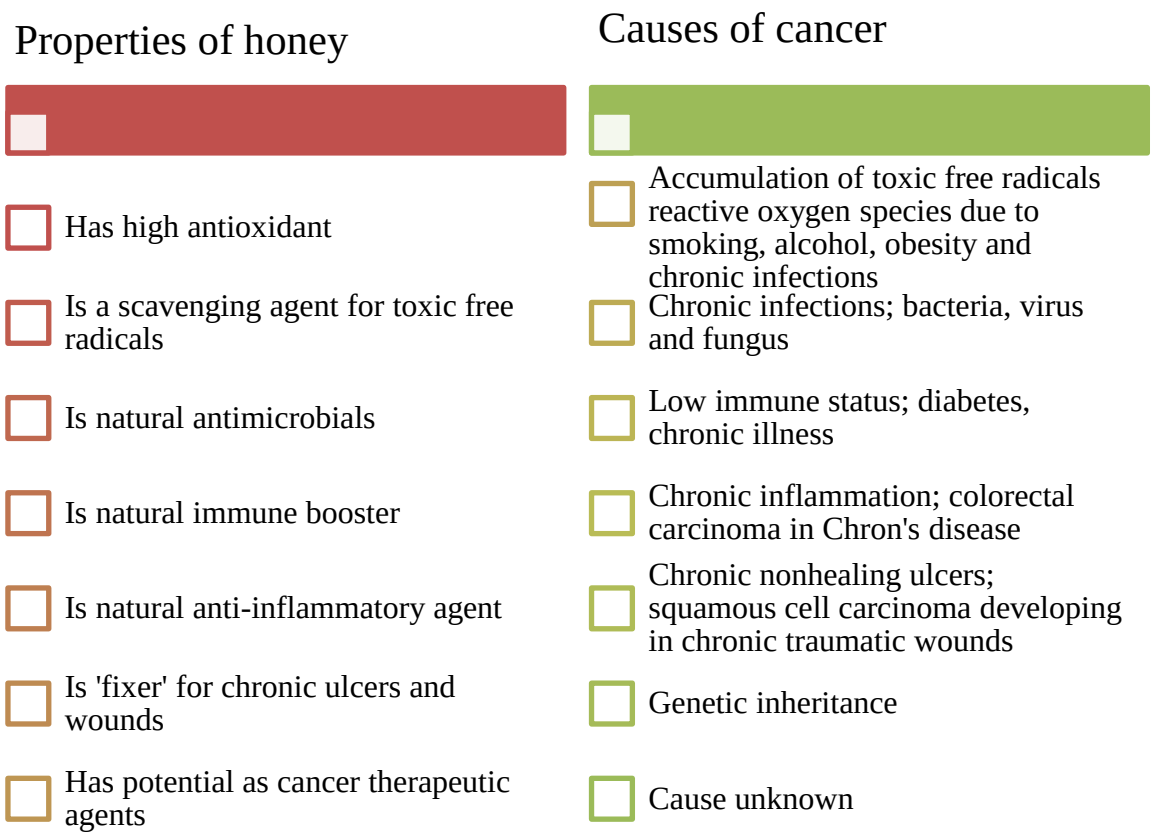


Figure 2.2: Inverse relationship between honey and cancer. Modified from: (Othman, 2012).

To compare physiochemical properties of stingless bee honey with normal honey, it is worthwhile taking a look into a study conducted in Thailand (Chuttong *et al.*, 2016). Their study had successfully analyzed 28 stingless bee honey samples and comparison was made to *A.mellifera* sp. honey standard. Results revealed that these stingless bee honeys had higher moisture content, high ash content, lower pH, higher acidity, lower diastase activity and lower total carbohydrates as compared to normal honeys' physiochemical composition. The

study served as the first study reporting physicochemistry from those south east Asian stingless bee species (Chuttong *et al.*, 2016).

Besides that, another study from Brazil comprised of physiochemical data from 152 stingless bee honey samples collected from year 1964 was evaluated for quality control proposal of several species of stingless bee honeys (Souza *et al.*, 2006). Summary of the physicochemical parameters taken into account are shown below in figure 2.3.

Bee species	Number of samples	Physico-chemical parameters[1]										
		pH	Free acidity (meq/K g honey)	Ash (g/100 g honey)	Diastase activity (DN)[2]	Electrical conductivity (mS/cm)	HMF (mg/Kg honey)	Invertase activity (IU)[3]	Nitrogen (mg/100 g honey)	Reducing sugars (g/100 g honey)	Sucrose (g/100 g honey)	Moisture (g/100 g honey)
Meliponini	152	3.81 (101)	44.8 (147)	0.34 (98)	6.7 (67)	2.34 (68)	14.4 (127)	48.7 (17)	58.31 (93)	66.0 (127)	2.3 (122)	26.7 (152)
Melipona spp.	97	3.82 (61)	41.8 (97)	0.20 (60)	3.1 (52)	2.62 (54)	16.0 (97)	56.3 (13)	40.78 (58)	69.1 (84)	2.2 (84)	27.2 (97)
other Meliponini	55	3.80 (40)	49.6 (50)	0.60 (38)	16.2 (15)	1.88 (14)	11.9 (30)	37.4 (4)	110.88 (35)	63.8 (43)	2.5 (38)	26.0 (55)
M. asilvai	11	3.27 (11)	41.6 (11)	-	-	3.63 (11)	2.4 (10)	-	-	68.9 (11)	4.7 (11)	29.5 (11)
M. compressipes	15	3.72 (10)	36.6 (15)	0.26 (13)	4.5 (13)	8.77 (1)	17.1 (15)	-	33.22 (13)	70.5 (13)	2.5 (13)	23.8 (15)
M. favosa	20	-	49.9 (20)	0.22 (20)	1.9 (20)	2.06 (6)	9.1 (21)	90.1 (6)	55.77 (20)	71.2 (20)	1.7 (20)	24.8 (20)
M. mandacaia	20	3.27 (20)	43.5 (20)	-	-	3.52 (20)	5.8 (20)	-	-	74.8 (20)	2.9 (20)	28.8 (20)
T. angustula	39	3.93 (31)	49.7 (34)	0.38 (29)	20.5 (8)	3.07 (8)	13.3 (14)	50.1 (3)	99.26 (3)	63.1 (34)	2.3 (29)	24.7 (39)

Figure 2.3: Summary of stingless bee honey composition (Souza *et al.*, 2006).

2.2.3 Polyphenols

Polyphenols comprise a wide variety of molecules with several hydrogenated substituents as well as other their functional derivatives on the aromatic rings; such as phenolic acids and phenolic alcohols. Polyphenols are divided into several classes, according to the number of phenolic rings present and to the structural elements connecting the rings to one another (Grassi *et al.*, 2009). It is mainly a supersaturated sugar solution, with more than 95% of its dry mass consisting of sugar, although different valuable nutrients such as vitamins, minerals, enzymes, flavouring organic compounds, free amino acids and numerous volatile compounds constitute minor components (Baroni *et al.*, 2006; Makawi *et al.*, 2009). However, it is this smaller fraction of the overall composition that is responsible for honey's organoleptic and nutritional properties (Manyi-Loh *et al.*, 2011). Honey composition varies due to the differences in plant types, climate and environmental conditions (Küçük *et al.*, 2007). Honey composition is tightly associated to its botanical source and also to the geographical area, because soil and weather determine melliferous flora. Depending on the botanical origin, honey could be classified as: (a) floral, when it is derived from the nectar of flowering plant, or (b) non-floral (honeydew) when it is derived from sweet deposits secreted by living parts of plants or excreted onto them by sap-sucking insects (Manyi-Loh *et al.*, 2011). The main group of phenolic includes derivatives of cinnamic acid, cumarins as well as flavonoids. Figure 2.4 shows chemical structure of polyphenols.

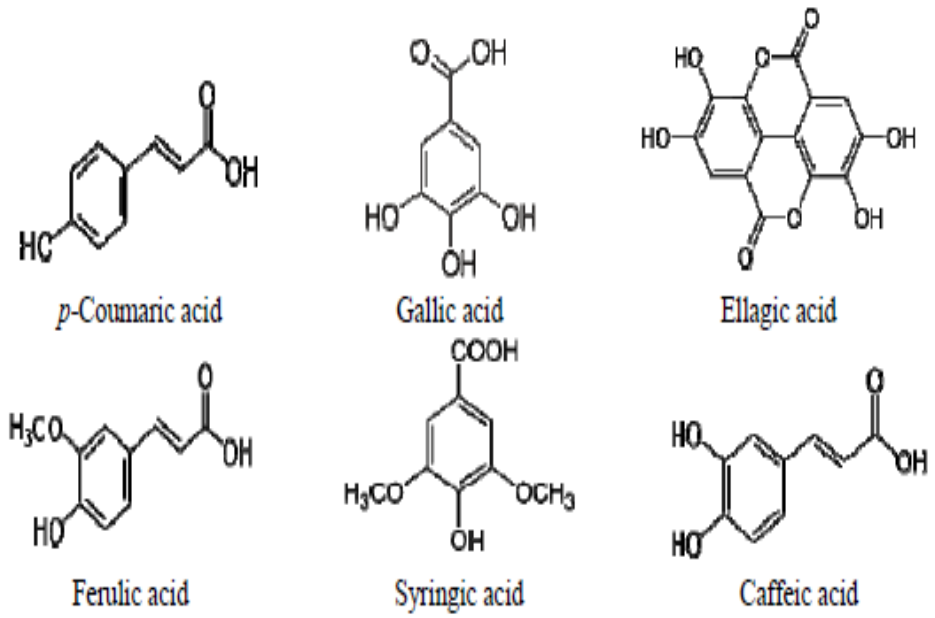


Figure 2.4: Chemical structures of phenolic acid (Erejuwa *et al.*, 2014).

2.2.3 (a) Mechanism known to attributing anti-cancer activity

Caffeic acid is an example of chemical compound consisting phenol group. Effect of caffeic acid and its ester group in honey has been proven to play a vital role in mechanism of antiproliferative and apoptotic effects on various malignant cells. Caffeic Acid Phenylethyl Ester (CAPE) undergoes oxidation by tyrosinase through two-electron oxidation to quinone and oxidized by HRP/H_2O_2 through one-electron oxidation to a semiquinone. CAPE toxicity towards SK-MEL-28 human melanoma cells was significantly enhanced by dicoumarol (DC), a diaphorase inhibitor, and 1-bromoheptane (BH), a GSH depleting agent. Ascorbic acid (AA), nicotinamide adenine dinucleotide (NADH), and glutathione (GSH) were depleted as a result of CAPE oxidation mediated by tyrosinase bioactivation.

Ethylenediamine, an o-quinone trap, reacts with CAPE o-quinone, preventing NADH and AA oxidation (Kuduguntil *et al*., 2010).

Rao *et al*. 1992, performed a detailed study by synthesizing caffeic acid esters and examined them against the 3, 2 -dimethyl-4- aminobiphenyl (DMAB, a colon and mammary carcinogen). Huang *et al.*1996, showed the strong repressive effect of caffeic acid phenethyl ester (CAPE), where inhibitory effect of CAPE was established on TPA-induced tumor promotion by topical application of CAPE in CD-I mice. The effects of caffeic acid phenethyl ester (CAPE) were first studied in carivcal carcinoma cells. CAPE showed cells growth inhibition by arresting the S phase. The arrest was associated with elevated expression of E2F1, Apaf-1 and reduced expression of Mc1-1. Besides that, cyclin A and E expression were increased while expression of cyclin B was decreased. The overall effects resulted in inhibition of the cell growth and thus led to cell cycle arrest (Kuo *et al.*, 2013).

In other study investigating cytotoxicity effect of CAPE on C6 glioma cells done, the study revealed that the glioma cells underwent internucleosomal DNA fragmentation upon 24hrs treatment with CAPE. Further study also reported that CAPE is capable to cause p53 dependent apoptosis in the glioma cells (Lee *et al.*, 2003). Besides, in-vitro biochemical mechanism of caffeic acid phenylethyl ester (CAPE) toxicity and eight hydroxycinnamic/caffeic acid derivatives in-vitro, using tyrosinase enzyme as a molecular target in human SK-MEL-28 melanoma cells was also investigated as shown below in Figure 2 (Kudugunti *et al.*, 2010).